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Biomarkers for Autism and for Gastrointestinal and Sleep Problems in  
Autism

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<b>14. ABSTRACT</b> The nighttime and daytime excretions of melatonin sulfate were not significantly different between typically developing (TD) toddlers and toddlers with autism spectrum disorders (ASDs). Sleep and gastrointestinal (GI) problems were not associated with lower melatonin excretion in TD or ASD toddlers and to a large extent these problems occurred at similar rates in the TD and ASD groups. The expected day-night differences in melatonin sulfate excretion, and in norepinephrine and epinephrine excretion, were seen in both groups. Thus, the lower melatonin production reported for older children with ASD does not appear to occur in toddlers with ASD.					
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## INTRODUCTION

The main objective of the proposed research was to compare the production of melatonin by young children with autism to that of typically developing children. Specifically, we have tested whether nighttime excretion rate of melatonin sulfate is markedly lower in the children with autism, whether there is a subgroup of children with autism having very low excretion; whether low nighttime excretion of melatonin sulfate is associated with sleep problems; and whether low daytime melatonin sulfate excretion is associated with gastrointestinal (GI) problems. We have also investigated the daytime and nighttime urinary excretion of norepinephrine, epinephrine and creatinine.

**Keywords:** urinary melatonin, toddlers, autism, sleep, gastrointestinal, norepinephrine, epinephrine

## Overall Project Summary

### Timeline

Problems with recruitment led to the need to extend the study past the original end date of September 30, 2013 to a revised end date of September 30, 2015. Requests for no-cost extensions were granted through September 30, 2015 in order that original total recruitment targets could be approached. Sample collection has been completed and biochemical analysis and data analysis have now been completed. The study has been re-approved by the Yale Human Investigation Committee through October 10, 2016.

### Results Summary

As described in more detail in the Body of the report, the results indicated the following:

- 1) The average nighttime and daytime excretions of melatonin sulfate were not substantially or significantly different between typically developing (TD) toddlers and toddlers with autism spectrum disorders (ASDs).
- 2) Sleep problems were not associated with lower melatonin excretion in TD or ASD toddlers.
- 3) GI problems were not associated with lower daytime excretion in either the TD or ASD group.
- 4) The expected day-night differences in melatonin sulfate excretion, and in norepinephrine and epinephrine excretion, were seen in both the TD and ASD groups.
- 5) Nighttime epinephrine excretion was observed to be significantly higher in the ASD group and this group difference was due to higher epinephrine excretion in those ASD individuals with sleep problems.
- 6) Caretaker report of items on the Sleep Behaviors and Gastrointestinal Symptoms Questionnaire indicated that to a large extent these behaviors and symptoms were similar across the TD and ASD groups.
- 7) However, there was a significantly higher reported frequency of a history of sleep problems and of a history of colic/vomiting/abdominal pains in the ASD group.

## BODY OF REPORT

### A. Overview

The data collected and measures obtained included parent report of sleep behaviors and gastrointestinal symptoms, urinary excretion rates for melatonin sulfate, norepinephrine, and epinephrine. Derived or calculated measures included inter-correlations among the

neurochemical excretion rates, correlations between the neurochemical excretion rates and rated clinical behaviors, and subgroup excretion rates in subgroups based on parent reported sleep behaviors and gastrointestinal symptoms.

## B. Parent Report of Sleep Behaviors and Gastrointestinal Symptoms

The parental report of sleep behaviors and gastrointestinal symptoms are tabulated in Table 1. The questionnaire that was used is included as report Appendix 1. Within the ASD and TD groups, the number of individuals with endorsement (positive) or non-endorsement (negative) are

**TABLE 1. SLEEP BEHAVIORS & GASTROINTESTINAL SYMPTOMS**

	ASD n=41		TD n=68		
GI ITEMS	ASD POS	ASD NEG	TD POS	TD NEG	FISHERS EXACT ONE-TAILED
COLIC RECENT	4 (9.8%)	37	0 (0%)	68	<b>0.0182</b>
COLIC HISTORY	9 (22%)	32	2 (2.9%)	66	<b>0.00229</b>
STOOL RECENT	13	28	22	46	0.559
STOOL HISTORY	7	34	6	62	0.163
GI DIAGNOSIS	5 (12%)	36	1 (1.5%)	67	<b>0.0274</b>
	<b>ASD</b>	<b>ASD</b>	<b>TD</b>	<b>TD</b>	
SLEEP ITEMS	MEAN	SD	MEAN	SD	t-test: t, df, p (2-tailed)
BEDTIME (pm) REC.	8.43	1.11	8.49	0.98	0.29, 107, .772
BEDTIME (pm) HIS.	8.38	1.18	8.27	0.86	0.52, 107, 0.604
LATENCY (min) REC.	21.96	23.14	18.38	20.17	0.85, 107, .397
LATENCY (min) HIS.	21.90	18.66	16.60	13.26	1.73, 107, .0865
NIGHT SLEEP (hrs) REC.	9.69	1.55	10.07	1.39	0.69, 107, .492
NIGHT SLEEP (hrs) HIS.	9.74	1.53	10.20	1.30	0.92, 107, .400
AWAKENINGS REC.	0.74	1.24	0.78	1.17	0.13, 107, .757
AWAKENINGS HIS.	0.88	1.75	0.95	1.20	0.38, 107, .705
NAPS (min) REC.	84.4	47.7	94.2	50.4	1.15, 107, .253
NAPS (min) HIS.	80.6	52.3	102.2	43.0	2.17, 107, <b>.0322</b>
SLEEP ITEMS	ASD POS	ASD NEG	TD POS	TD NEG	FISHERS EXACT ONE-TAILED
AWAKENINGS REC. +/-	17	24	30	38	0.843
AWAKENINGS HIST. +/-	19	22	40	28	0.237
SLEEP PROB REC.	10	31	10	58	0.307
SLEEP PROB HIS.	17 (41%)	24	13 (19%)	55	<b>0.0151</b>
NIGHTMARES HIS.	13	28	13	55	0.166
BREATHING PROB. HIS.	14 (34%)	27	10 (15%)	58	<b>0.0329</b>
JERK/TALK HIS.	10	31	14	54	0.812

given for each qualitative item. Although several items tended to be endorsed more often in the ASD group (e.g. recent colic 4/41 versus 0/68; GI diagnosis 5/41 versus 1/68; history of sleep problems 17/41 versus 13/55; see highlighted p values), the only test that remained significant

after correction for the number of comparisons was the greater occurrence of a history of colic in the ASD group (9/41 (22%) versus 2/ 68 (2.9%),  $p=0.00229$ ). A history of sleep problems was endorsed more often in the ASD group versus the TD group (41% versus 19%,  $p=0.0151$ ), however this was considered a trend level finding given the need for multiple comparison correction. The quantitative sleep variables included bedtimes, time to fall asleep (latency), total nighttime sleep time, number of awakenings, and daytime naps. None of the quantitative sleep variables differed significantly across the ASD and TD groups, although a lower mean total daytime naptime was observed in the ASD group (80.6 minutes versus 102 minutes,  $p=0.032$ ).

### C. Biochemical Measures: Summary Statistics and Inter-Correlations

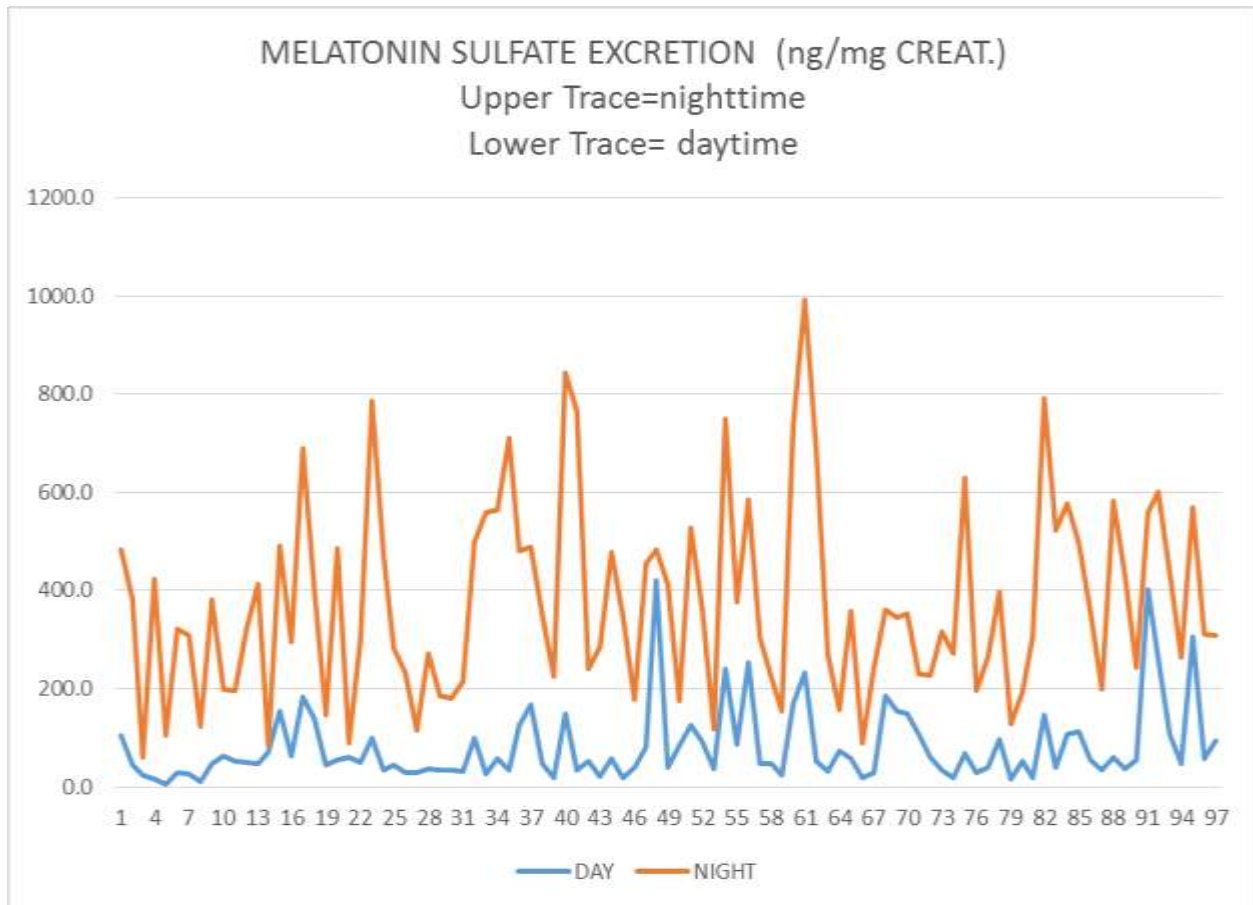
The biochemical data (mean, standard deviation, median) are tabulated in Table 2. All neurochemical measures are expressed as per mg of creatinine in order to account for the dilution of each urine sample. In the absence of such a correction for urinary dilution, an individual's value would be unduly affected by their urine volume and this is in turn largely determined by how much water they had consumed. Both daytime and nighttime melatonin sulfate (MEL) excretion rates were similar in the ASD group compared to the typically developing (TD) group. Significantly lower daytime norepinephrine (NE) excretion and significantly higher nighttime epinephrine (EPI) excretion was observed in the ASD group compared to the TD group. Other measures were similar across the two groups.

**TABLE 2. SUMMARY OF BIOCHEMICAL MEASURES**

URINARY MEASURE	ASD	ASD	ASD	ASD	NC	NC	NC	NC	STATISTICS (z, p)
	mean	SD	med.	n	mean	SD	med.	n	Mann-Whitney U-test
<b>MEL DAY</b> (ng/mg creat.)	<b>93.1</b>	86.9	59.1	37	<b>72.6</b>	70.0	48.0	62	0.93, 0.176 1-tail
<b>MEL NIGHT</b> (ng/mg creat.)	<b>292</b>	166	252	37	<b>293</b>	170	272	63	0.27, 0.394 1-tail
<b>NE DAY</b> (ng/mg creat.)	<b>29.1</b>	21.6	24.5	41	<b>39.9</b>	25.7	36.2	66	2.91, <b>0.0036</b> 2-tail
<b>NE NIGHT</b> (ng/mg creat.)	<b>20.7</b>	15.2	17.6	41	<b>27.2</b>	22.0	21.8	66	1.39, 0.165 2-tail
<b>EPI DAY</b> (ng/mg creat.)	<b>10.6</b>	9.8	8.3	41	<b>10.9</b>	9.9	8.0	66	0.1, 0.920 2-tail
<b>EPI NIGHT</b> (ng/mg creat.)	<b>6.9</b>	9.7	5.0	41	<b>4.7</b>	7.2	2.3	66	2.77, <b>0.0056</b> 2-tail
<b>CREAT. DAY</b> (mg/mL)	<b>0.199</b>	0.148	0.169	41	<b>0.206</b>	0.162	0.160	66	0.07, 0.944 2-tail
<b>CREAT. NIGHT</b> (mg/mL)	<b>0.222</b>	0.122	0.174	41	<b>0.274</b>	0.155	0.235	66	1.36, 0.174 2-tail

The three neurochemical measures all showed the expected day-night differences. As seen in Table 2 and as illustrated strikingly in Figure 1 for all 97 subjects with paired day-night samples, melatonin sulfate excretion was higher at night than during the day in both the TD

FIGURE 1.



group (Wilcoxon Signed-Rank Test,  $z=6.37$ ,  $p<0.0001$ ) and the ASD group ( $z=4.82$ ,  $p<0.0001$ ). The three- to four-fold higher mean nighttime versus daytime excretion rate is consistent with the well-established nocturnal activation of pineal synthesis and release of melatonin. Daytime excretion rates of norepinephrine (TD group  $z=5.58$ ,  $p<0.0001$ ; ASD group  $z=3.02$ ,  $p=0.0013$ ) and of epinephrine (TD group  $z=4.74$ ,  $p<0.0001$ ; ASD group  $z=2.46$ ,  $p=0.0069$ ) were both higher than during the night, consistent with the known daytime activation of the sympathetic nervous system and the adrenomedullary system.

The calculated inter-correlations for the biochemical measures are presented in Table 3. The only significant correlations observed were between nighttime and daytime norepinephrine (NE) excretion and between nighttime and daytime creatinine (Creat.) excretion. The significant positive correlations between daytime and nighttime excretion of a particular biochemical are expected [the absence such a correlation with epinephrine (EPI) is apparently due to its nighttime production being associated with sleep problems -as will be discussed]. The absence of significant correlations across different biochemical indicates that the systems (pineal melatonin, gut melatonin, sympathetic nervous system, adrenomedullary system) do not strongly influence

one another. The low and non-significant correlations between the neurochemical measures expressed as per mg of creatinine and creatinine itself indicates that creatinine is appropriately correcting for urinary dilution/volume (e.g., if a high creatinine value did not actually indicate a more concentrated urine, correcting for urine concentration –by dividing values by creatinine levels- would lead to neurochemical values being negatively correlated with creatinine).

**TABLE 3. CORRELATION MATRIX OF BIOCHEMICAL MEASURES  
(Spearman Rank Correlation r values)**

	NE day	EPI day	Creat. day	NE night	EPI night	Creat. night	MEL day	Mel night
NE day	1	0.383	-0.024	0.55	-0.033	0.105	-0.255	0.09
EPI day	0.383	1	-0.09	0.387	0.063	0.028	-0.132	0.108
Creat. day	-0.024	-0.09	1	0.081	0.006	0.536	-0.097	-0.124
NE night	<b>0.55*</b>	0.387	0.081	1	0.03	-0.061	0.038	0.204
EPI night	-0.033	0.063	0.006	0.03	1	-0.05	0.126	0.374
Creat. night	0.105	0.028	<b>0.536 ^</b>	-0.061	-0.05	1	-0.053	-0.183
MEL day	-0.255	-0.132	-0.097	0.038	0.126	-0.053	1	0.123
Mel night	0.09	0.108	-0.124	0.204	0.374	-0.183	0.123	1

\*p=0.00042 uncorrected; p=0.012 Bonferonni corrected

^p=0.00060 uncorrected; p=0.018 Bonferonni corrected

#### **D. Comparisons of ASD Subgroups**

The planned comparisons between ASD subgroups defined on the basis of parent reported are given in Table 4. Given the trend to higher nighttime epinephrine excretion we observed in the ASD versus the TD group and in order to preserve power, this was considered the primary hypothesis for the subgroup (endorsed/non-endorsed) comparisons. The p values for the other secondary hypotheses/tests should be corrected for the number of tests (multiply by 4). Other completely exploratory comparisons made between ASD subgroups were all non-significant even without any correction for multiple comparisons.



**TABLE 4. COMPARISONS OF URINARY EXCRETION RATES (ng/mg creat) IN ASD SUBGROUPS DEFINED ON THE BASIS OF SLEEP & GI QUESTIONNAIRE ITEMS**

	SUBGROUPING ITEM	N+/N-	Item Endorsed (+) mean±SD, median	Item Not Endorsed mean±SD, median	M-W U-test Z, p (one tail)
<b>EPI night</b>	Sleep problem history	17/24	7.57±6.17, 5.32	4.03±2.39, 4.08	1.7, <b>0.045</b>
<b>Mel night</b>	Sleep problem history	14/23	287±193, 215	293±152, 262	0.81, 0.209
<b>MEL day</b>	Abdom. Pain/ Colic /vomiting history	9/28	91.9±70.4, 73.4	93.5±92.7, 57.1	1.4, 0.081
<b>MEL night</b>	Abdom. Pain/ Colic /vomiting history	9/28	246±110, 300	307±180, 244	0.12, 0.452

#### **E. Neurochemical and ASD Behavioral Correlations**

Spearman Rank correlations were performed between the six neurochemical measures and the three standardized severity scores (scored 1 to 10) of the Autism Diagnostic Observation Schedule (ADOS; Western Psychological Services, Los Angeles, CA) obtained using the Toddler Module or Module 2 of the ADOS. Standardized severity scores of the Social Affect (SA) domain, the Restricted and Repetitive Behaviors (RRB) domain and the Combined SA & RRB domains were based on raw score ratings by ADOS trained raters of toddlers in the ASD group. All 18 (6 x 3) calculated correlations were low and none approached significance even without any correction for multiple testing.

#### **F. Demographic Data**

Mean (±SD, median) ages in months in the ASD (N=41) and TD (68) groups were 27.4 (±6.7, 24) and 24.8 (±5.7, 24), respectively (Student t=2.12, p=0.036). Of the 41 ASD subjects, 35 were white and 6 non-white, with none reporting a seizure history; of the 68 TD subjects, 58 were white and 10 non-white, with none reporting a seizure history. The racial proportions were not significantly different between the groups (chi-square p=0.79). Although the mean age in the ASD group was 3 months older than the TD group, none of the neurochemical measures showed a significant correlation with age and therefore age was not used as a covariate in the data analyses. Reassignment of several of the initial ASD group assignments to a Global Developmental Delay category resulted in a final ASD group membership of 41, rather than the target of 45. However, the oversampling of the TD group (N=68 actual, target N=45) resulted in somewhat greater power for the group comparisons than obtainable with two groups of 45 each (the statistical power with groups of sizes 68 and 45 is equal to that of two equal-sized groups of 51).

## KEY RESEARCH ACCOMPLISHMENTS

- 1) *We have made the first measurement of urinary excretion of melatonin sulfate in toddlers diagnosed with ASD and now report that group rates of excretion were similar in ASD and typically developing (TD) groups.*

A major research accomplishment of the study was the initial measurement of urinary excretion of melatonin sulfate in toddlers diagnosed with ASD. Measurement of this index of circadian rhythms in relatively large groups of toddlers classified as ASD or as TD provided the first comparative assessment of melatonin physiology in toddlers with ASD and in TD toddlers. Unlike prior findings of lower melatonin production in older children, adolescents and young adults with ASD (1, 2), the production of melatonin was similar in the ASD and TD groups. This basic finding suggests that in most individuals an alteration in melatonin production does not play a role in the early development of ASD, but rather that either the alterations in melatonin occur later in the developmental course of ASD or that the differences arise as an epiphenomenon or consequence of ASD.

- 2) *We have made the first measurements of urinary excretion of norepinephrine and epinephrine in toddlers diagnosed with ASD.*

Measurement of norepinephrine and epinephrine (adrenaline) in relatively large groups of ASD and TD toddlers has provided the first comparative assessment of sympathetic nervous system noradrenaline production and adrenomedullary epinephrine production in toddlers with ASD and in TD toddlers. As seen previously in older children, adolescents and young adults with ASD, the excretion rates seen for norepinephrine were somewhat lower in the ASD group (3). However, as will be discussed, while daytime excretion of epinephrine was similar in the two groups, nighttime epinephrine excretion was substantially and significantly greater in the ASD group.

- 3) *We have made the first comparison of the diurnal (day-night) variation in the production of melatonin, norepinephrine and epinephrine in TD and ASD toddlers.*

As previously observed in older children, adolescents and young adults with ASD, the ratio of daytime and nighttime production of melatonin (1, 2) and norepinephrine (3) were similar in the ASD and TD groups. Thus, although prior studies have consistently found lower melatonin production in ASD groups, they did find the typical greater nighttime production relative to daytime production, and in this way the present findings are consistent with prior studies. Although the expected greater daytime versus nighttime production of epinephrine was observed in ASD individuals, as mentioned, nighttime production of epinephrine was greater in the ASD group.

- 4) *We have performed the first investigation in toddlers with ASD of possible associations between melatonin, norepinephrine and epinephrine production and of sleep behaviors/problems, gastrointestinal (GI) problems, and ADOS-rated domain severity.*

This first attempt to determine whether sleep and GI problems might be related to differences in melatonin was based on melatonin's prominent role in biological rhythms and sleep onset (4) and on its important role in gut physiology (5, 6). Norepinephrine and epinephrine were of interest in sleep due to their reported marked diurnal rhythm (3). No significant associations were seen

between melatonin or norepinephrine and the range of problems that were asked about. However, nighttime epinephrine production was substantially and significantly higher in the subgroup of ASD individuals with a reported history of sleep problems compared to those ASD individuals without such problems. In fact, the higher nighttime epinephrine production observed in the total ASD group was due entirely to higher production in the subgroup with a history of sleep problems.

## CONCLUSIONS

A major conclusion of the study was that nighttime and daytime excretions of melatonin sulfate were not significantly different between typically developing (TD) toddlers and toddlers with autism spectrum disorders (ASDs). This is in contrast to multiple reports of lower melatonin production in older children with ASDs. A second main finding was that sleep problems were not associated with lower melatonin excretion in TD or ASD toddlers. Taken together, these results indicate that melatonin is unlikely to be involved in the underlying neurodevelopmental etiology of ASDs or to play a role in sleep problems often associated with ASD. This further suggests that assessment of melatonin production is unlikely to provide a useful predictor or biomarker of ASD or of sleep problems in ASD. Similarly, no association was observed between GI problems and daytime melatonin sulfate excretion. Because most daytime melatonin appears to arise from the intestine (6), this suggests that gut melatonin does not play a role in the GI problems often associated with ASDs.

The divergence between the melatonin findings in toddlers versus those previously reported in older subjects raises several issues including a.) whether the findings in both age groups can be replicated, b.) whether there is an identifiable ontogeny for the lower melatonin production seen in older ASD children, and c.) whether the apparently lower melatonin production in older individuals plays a causative role in ASD or if the lower production is a consequence of having an ASD.

As observed in several prior studies of diurnal rhythms in older individuals with ASDs, the expected within-individual day-night differences were seen for melatonin, norepinephrine and epinephrine, indicating that altered biological rhythms do not play a major role in the early stages of ASDs. However, the finding of higher nighttime epinephrine excretion in individuals with ASD and concomitant sleep problems suggests that altered adrenomedullary functioning might be involved in the sleep problems and that further research examining the possible role of adrenal functioning in this area is warranted.

## Publications, Abstracts and Presentations

Abstract submitted to the International Society for Autism Research (INSAR) and accepted for presentation at the 2016 annual meeting (May 11-14, 2016, Baltimore, MD):

*ABSTRACT #22543*

*Diurnal Production and Behavioral Associations of Melatonin and Other Neurohormones in Autism Spectrum Disorders*

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### **Inventions, Patents and Licenses**

None to date

### **Reportable Outcomes**

The results of the study are reportable and will be submitted for publication in peer reviewed journals.

### **Other Achievements**

Demonstrated feasibility of diaper collection from individuals with ASDs for use in biochemical analyses.

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## Appendix 1. Sleep Behaviors & Gastrointestinal Symptoms Questionnaire

Your Name: \_\_\_\_\_ Relationship to Child: \_\_\_\_\_ Date: \_\_\_\_\_

Child's Name: \_\_\_\_\_ Child's Date of Birth: \_\_\_\_\_

### Questionnaire (please circle the one best answer)

Last week, did your child vomit, have abdominal pains or colic? **No** **Once or Twice** **3 Times or More**

Has your child ever had a problem with vomiting, abdominal pains or colic for more than 2 weeks? **Yes** **No**

Last week, did your child have constipation, diarrhea or abnormal stools? **Never** **Once or Twice** **3 Times or More**

Has your child ever had a problem with diarrhea, constipation, or abnormal stools for more than 2 weeks? **Yes** **No**

Has your child ever been diagnosed with a gastrointestinal disorder by a gastroenterologist or pediatrician? **Yes** **No**

At what time is your child put to bed at night: \_\_\_\_\_  
Usually Last night

How long (minutes) does it take your child to fall asleep after being put to bed: \_\_\_\_\_  
Usually Last night

How many hours does your child sleep at night (between 7PM & 7AM): \_\_\_\_\_  
Usually Last night

How many times does your child wake up at night: \_\_\_\_\_  
Usually Last night

How many minutes does your child nap during the day: \_\_\_\_\_  
Usually Yesterday

Last night, was your child's sleep: **A serious problem** **A small problem** **Not a problem**

Do you think your child's sleep is usually: **A serious problem** **A small problem** **Not a problem**

Does your child have nightmares or sleep walk: **often** **occasionally** **rarely** **never**

Does your child snore or have breathing problems during sleep: **often** **occasionally** **rarely** **never**

Does your child jerk repeatedly, grind teeth, or talk during sleep: **often** **occasionally** **rarely** **never**

{For last 3 questions: **often**= once a week or more; **occasionally**= 1-3 times a month; **rarely**= less than once a month }